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(54) Title: ATTENUATED RESPIRATORY SYNCYTIAL VIRUS VACCINE COMPOSITIONS

(57) Abstract

The present invention provides vaccine compositions of attenuated respiratory syncytial virus (RSV). More particularly, the attenuated virus may be a derivative of RSV which has been incompletely attenuated by cold-passage or introduction of mutations which produce virus having a temperature sensitive (ts) or cold adapted (ca) phenotype. The invention also provides methods for stimulating the immune system of an individual to induce protection against respiratory syncytial virus by administration of attenuated RSV. The invention also provides pure cultures of attenuated RS virus, wherein the virus has been more completely attenuated by the further derivatization of previously identified incompletely attenuated ts or cp mutants.

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ATTENUATED RESPIRATORY SYNCYTIAL VIRUS VACCINE COMPOSITIONS

Background of the Invention

Respiratory syncytial (RS) virus infection of humans ranges from asymptomatic to severe respiratory tract disease. In infants and children, RS virus (RSV) is regarded as one of the most important causes of lower respiratory tract disease in all geographic areas of the world. RS virus outranks all other microbial pathogens as a cause of pneumonia and bronchiolitis in infants under one year of age, and is a major cause of fatal respiratory tract disease in these infants. Virtually all children are infected by two years of age. Reinfection occurs with appreciable frequency in older children and young adults. (Chanock et al., in Viral Infections of Humans, 3d ed., A.S. Evans, ed., Plenum Press, N.Y. (1989)). Although most healthy adults do not have serious disease due to RS virus infection, elderly patients and immunocompromised individuals are more likely to have severe and possibly life-threatening infections.

Treatment of RSV infection has been problematic. Small infants have diminished serum and secretory antibody responses to RSV antigens and thus suffer more severe infections, whereas cumulative immunity appears to protect older children and adults against more serious forms of the infection. One antiviral compound, ribavirin, has shown promise in the treatment of severely infected infants, although there is no indication that it shortens the duration of hospitalization or diminishes the infant's need for supportive therapy.

The mechanisms of immunity in RSV infection have recently come into focus. Secretory antibodies appear to be most important in protecting the upper respiratory tract, whereas high levels of serum antibodies are thought to have a major role in resistance to RSV infection in the lower respiratory tract. Purified human immunoglobulin containing a high titer of neutralizing antibodies to RSV may prove useful in immunotherapeutic approaches for serious lower respiratory tract disease in infants and young children. Immune globulin preparations, however, suffer from several disadvantages, such as the possibility of transmitting blood-borne viruses and difficulty and expense in preparation and storage.

Despite an urgent need for an effective vaccine against RS virus, particularly for infants and young children, previous attempts to develop a safe and effective vaccine have met with failure. A formalin-inactivated virus vaccine tested in

the mid-1960s failed to protect against RS virus infection or disease. Instead, disease was exacerbated during subsequent infection by RS virus. Kim et al., Am. J. Epidemiol. 89:422-434, Chin et al., Am J. Epidemiol. 89:449-463 (1969); Kapikian et al., Am. J. Epidemiol. 89:405-421 (1969).

To circumvent the problems attendant with the inactivated vaccines and the possible alteration of neutralization epitopes, efforts were directed to developing attenuated RS mutants. Friedewald et al., J. Amer. Med. Assoc. 204:690-694 (1968) reported the production of a low-temperature passaged mutant of RS virus which appeared to possess sufficient attenuation to be a candidate vaccine. This mutant exhibited a slight increased efficiency of growth at 26°C compared to its wild-type parental virus, but its replication was neither temperature sensitive nor significantly cold-adapted. The cold-passaged mutant, however, was attenuated for adults. Although satisfactorily attenuated and immunogenic for infants and children who had been previously infected with RSV (i.e., seropositive individuals), the mutant retained a low level virulence for the upper respiratory tract of seronegative infants. This RSV mutant had been passaged in bovine kidney cell culture at low temperature (26°C) and as a consequence it acquired host range attenuating mutations. The acquisition of these mutations allowed the mutant to replicate efficiently in bovine tissue, whereas these same mutations restricted growth of the mutant in the human respiratory tract compared to its RSV strain A2 parent.

Similarly, Gharpure et al., J. Virol. 3:414-421 (1969) reported the isolation of temperature sensitive (ts) mutants which also were promising vaccine candidates. One mutant, ts-1, was evaluated extensively in the laboratory and in volunteers. The mutant produced asymptomatic infection in adult volunteers and conferred resistance to challenge with wild-type virus 45 days after immunization. Again, while seropositive infants and children underwent asymptomatic infection, seronegative infants developed signs of rhinitis and other mild symptoms. Furthermore, instability of the ts phenotype was detected, although virus exhibiting a partial or complete loss of temperature sensitivity represented a small proportion of virus recoverable from vaccinees, and was not associated with signs of disease other than mild rhinitis.

The studies thus revealed that among the cold-passaged and temperature sensitive strains some were underattenuated and caused mild symptoms of disease in some vaccinees, particularly seronegative infants, while others were overattenuated and failed to replicate sufficiently to elicit protective immune responses. (Wright et al., Infect. Immun. 37:397-400 (1982)). The genetic instability that allowed candidate

vaccine mutants to lose their temperature-sensitive phenotype was also a disconcerting discovery. See generally, Hodes et al., Proc. Soc. Exp. Biol. Med. 145:1158-1164 (1974), McIntosh et al. Pediatr. Res. 8:689-696 (1974), and Belshe et al., J. Med. Virol. 3:101-110 (1978).

Abandoning the attenuated RS virus vaccine approach, investigators tested potential subunit vaccine candidates using purified RS virus envelope glycoproteins from lysates of infected cells. The glycoproteins induced resistance to RS virus infection in the lungs of cotton rats, Walsh et al., J. Infect. Dis. 155:1198-1204 (1987), but the antibodies induced had very weak neutralizing activity and immunization of rodents with purified subunit vaccine led to disease potentiation (Murphy et al., Vaccine 8:497-502 (1990)).

Vaccinia virus recombinant-based vaccines which express the F or G envelope glycoprotein have also been explored. These recombinants express RSV glycoproteins which are indistinguishable from the authentic viral counterpart, and small rodents infected intradermally with the vaccinia-RSV F and G recombinant viruses developed high levels of specific antibodies that neutralized viral infectivity. Indeed, infection of cotton rats with vaccinia-F recombinants stimulated almost complete resistance to replication of RSV in the lower respiratory tract and significant resistance in the upper tract. Olmsted et al., Proc. Natl. Acad. Sci. USA 83:7462-7466 (1986). However, immunization of chimpanzees with vaccinia F and vaccinia G recombinant provided almost no protection against RSV challenge (Collins et al., Vaccine 8:164-168 (1990)). This led to the conclusion that this approach was not likely to yield a successful vaccine.

While investigators examined several different approaches to producing an effective and safe RS vaccine over the years, RS virus has remained the most common cause of severe viral lower respiratory tract disease in infants and children. Consequently, an urgent need remains for a safe vaccine that is able to prevent the serious illness in this population that often requires hospitalization, and to prevent disease in other individuals. Quite surprisingly, the present invention fulfills these and other related needs.

Summary of the Invention

The present invention provides vaccine compositions of attenuated respiratory syncytial virus. The attenuated virus is provided in an amount sufficient to induce an immune response in a human host, in conjunction with a physiologically acceptable carrier and may optionally include an adjuvant to enhance the immune response of the host. The invention contemplates several distinct antigenic subgroups of attenuated RS virus which are derived from incompletely attenuated RS virus and which possess properties heretofore not exhibited by attenuated RS viruses previously reported in the literature. In one embodiment thereof, the attenuated virus of the invention comprises host range restricted RS virus incompletely attenuated by cold-passage (cpRSV) into which at least one or more additional mutations are introduced to produce a virus and its progeny having a temperature sensitive (ts) phenotype, which are hereinafter designated cptsRSV. In another embodiment, host-range restricted RS virus incompletely attenuated by cold-passage (cpRSV) is cold adapted (ca) by passage at increasingly reduced temperatures to introduce additional growth restriction mutations. In yet another embodiment, incompletely attenuated RSV ts mutants, such as RSV ts-4 and ts-1,NG1 are further attenuated by introduction of additional mutations. The attenuated derivatives of the ts or cp strains are produced in several ways, but preferably by introduction of additional temperature sensitive mutations by chemical mutagenesis, by further passage in culture at attenuating temperatures of 20-24°C, or by introduction of small plaque (sp) mutations and selection of derivatives which are more restricted in replication than the incompletely attenuated parental mutant strain. The attenuated virus of the invention belongs to either antigenic subgroup A or B, and virus from both subgroups may conveniently be combined in vaccine formulations for more comprehensive coverage against prevalent RSV infections. The vaccine will typically be formulated in a dose of from about 10^3 to 10^6 plaque-forming units (PFU) or more for maximal efficacy.

In other embodiments, the invention provides methods for stimulating the immune system of an individual to induce protection against respiratory syncytial virus. These methods comprise administering to the individual an immunologically sufficient amount of RSV which has been attenuated by introducing mutations that specify the ts, ca, and/or sp phenotype into RSV which was originally incompletely attenuated by ts mutation(s) or by passage at cold temperature, e.g., 26°C. In view of the potentially serious consequences of RSV infection in neonates, seronegative and seropositive infants and young children, and the elderly, these individuals will typically

benefit most from immunization according to the present methods. In most instances the attenuated RS virus is administered to the respiratory tract of the individual, preferably intranasally by aerosol or droplet application.

In yet other embodiments, the invention provides pure cultures of attenuated RS virus, wherein the virus has been more completely attenuated by the further derivatization of previously identified ts or cp mutants. The attenuated virus is capable of eliciting a protective immune response in an infected human host yet is sufficiently attenuated so as to not cause unacceptable symptoms of severe respiratory disease in the immunized host. The attenuated virus may be present in a cell culture supernatant, isolated from the culture, or partially or completely purified. The virus may also be lyophilized, and can be combined with a variety of other components for storage or delivery to a host, as desired.

Description of the Specific Embodiments

The present invention provides RS virus suitable for vaccine use in humans. The RS virus described herein is produced by introducing additional mutations into incompletely attenuated strains of ts or cp RS virus. The mutations are introduced into the strains during virus growth in cell cultures to which a chemical mutagen has been added, by selection of virus that has been subjected to passage at suboptimal temperature in order to introduce growth restriction mutations, or by selection of mutagenized virus that produces small plaques in cell culture.

Thus, the vaccine of the invention comprises the attenuated RV virus and a physiologically acceptable carrier. The vaccine is administered in an immunogenically sufficient amount to an individual in need of immunological protection against RS virus, such as, e.g., an infant, child, the elderly, or adult candidates for immunosuppressive therapies. The vaccine elicits the production of an immune response that is protective against serious lower respiratory tract disease, such as pneumonia and bronchiolitis when the individual is subsequently infected with wild-type RS virus. While the naturally circulating virus is still capable of causing infection, particularly in the upper respiratory tract, there is a very greatly reduced possibility of rhinitis as a result of the vaccination and possible boosting of resistance by subsequent infection by wild-type virus. Following vaccination, there are detectable levels of host engendered serum and secretory antibodies which are capable of neutralizing homologous (of the same subgroup) wild-type virus in vitro and in vivo. In many

instances the host antibodies will also neutralize wild-type virus of a different, non-vaccine subgroup. To achieve higher levels of cross-protection, i.e., against heterologous strains of another subgroup, it is preferred to vaccinate individuals with attenuated RS virus from at least one predominant strain of both subgroups A and B.

The attenuated virus which is a component of the vaccine is in an isolated and typically purified form. By isolated is meant to refer to attenuated modified RS virus which is in other than the native environment of wild-type virus, such as the nasopharynx of an infected individual. More generally, isolated is meant to include the attenuated virus as a heterologous component of a cell culture or other system. For example, attenuated RS virus of the invention may be produced by an infected cell culture, separated from the cell culture and added to a stabilizer which contains other non-naturally occurring RS viruses, such as those which are selected to be attenuated by means of resistance to neutralizing monoclonal antibodies to the F-protein, as described in co-filed U.S. patent application attorney docket 15280-11-2, the disclosure of which is expressly incorporated herein by reference.

The attenuated RS virus of the present invention exhibits a very substantial diminition of virulence when compared to wild-type virus that is circulating naturally in humans. The attenuated virus is sufficiently attenuated so that symptoms of infection will not occur in most immunized individuals. In some instances the attenuated virus may still be capable of dissemination to unvaccinated individuals. However, its virulence is sufficiently abrogated such that severe lower respiratory tract infections in the vaccinated or incidental host do not occur.

The level of attenuation may be determined by, for example, quantifying the amount of virus present in the respiratory tract of an immunized host and comparing the amount to that produced by wild-type RS virus or other attenuated RS viruses which have been evaluated as candidate vaccine strains. For example, the attenuated virus of the invention will have a greater degree of restriction of replication in the upper respiratory tract of a highly susceptible host, such as a chimpanzee, compared to the levels of replication of wild-type virus, e.g., 10- to 1000-fold less. Also, the level of replication of the attenuated RSV vaccine strain in the upper respiratory tract of the chimpanzee should be less than that of the incompletely attenuated RSV A2 ts-1 mutant. In order to further reduce the development of rhinorrhea, which is associated with the replication of virus in the upper respiratory tract, an ideal vaccine candidate virus should exhibit a restricted level of replication in both the upper and lower respiratory tract. However, the attenuated viruses of the invention must be sufficiently infectious and immunogenic in humans to confer protection in vaccinated individuals. Methods

for determining levels of RS virus in the nasopharynx of an infected host are well known in the literature. Specimens are obtained by aspiration or washing out of nasopharyngeal secretions and virus quantified in tissue culture or other by laboratory procedure. See, for example, Belshe et al., J. Med. Virology 1:157-162 (1977), Friedewald et al., J. Amer. Med. Assoc. 204:690-694 (1968); Gharpure et al., J. Virol. 3:414-421 (1969); and Wright et al., Arch. Ges. Virusforsch. 41:238-247 (1973). The virus can conveniently be measured in the nasopharynx of host animals, such as chimpanzees.

To produce a satisfactorily attenuated derivative virus of the present invention mutations are introduced into a parental viral strain which has been incompletely or partially attenuated, such as the ts-1 or ts-4 mutant, or cpRSV. For virus of subgroup A, the incompletely attenuated parental virus is preferably ts-1 or ts-1 NG-1 or cpRSV, which are mutants of the A2 strain of subgroup A, or derivatives or subclones thereof.

Partially attenuated mutants of the subgroup B virus can be produced by biologically cloning wild-type subgroup B virus in an acceptable cell substrate and developing cold-passage mutants thereof, subjecting the virus to chemical mutagenesis to produce ts mutants, or selecting small plaque mutants thereof. The various selection techniques may also be combined to produce the partially attenuated mutants of subgroup A or B which are useful for further derivatization as described herein.

Once the desired partially attenuated parental strain(s) is/are selected, further attenuation sufficient to produce a vaccine acceptable for use in humans according to the present invention may be accomplished in several ways as described herein.

According to the present invention the cp mutant can be further mutagenized in several ways. In one embodiment the procedure involves subjecting the partially attenuated virus to passage in cell culture at progressively lower, attenuating temperatures. For example, whereas wild-type virus is typically cultivated at about 34-35°C, the partially attenuated mutants are produced by passage in cell cultures (e.g., primary bovine kidney cells) at suboptimal temperatures, e.g., 26°C. These mutants have slight but definite evidence of cold adaptation (ca), i.e., increased efficiency of growth at 26°C compared to its wild-type parental virus, but typically are not ts. Thus, in one method of the present invention the cp mutant or other partially attenuated strain, e.g., ts-1 or sp, is adapted to efficient growth at a lower temperature by passage in MRC-5 or Vero cells, down to a temperature of about 20-24°C, preferably 20-22°C.

This selection of mutant RS virus during cold-passage substantially eliminates any residual virulence in the derivative strains as compared to the partially attenuated parent.

In another embodiment of the invention the incompletely attenuated strains are subjected to chemical mutagenesis to introduce ts mutations or, in the case of viruses which are already ts, additional ts mutations sufficient to confer increased stability of the ts phenotype on the attenuated derivative. Means for the introduction of ts mutations into RS virus include replication of the virus in the presence of a mutagen such as 5-fluorouridine or 5-fluorouracil in a concentration of about 10^{-3} to 10^{-5} M, preferably about 10^{-4} M, or exposure of virus to nitrosoguanidine at a concentration of about 100 µg/ml, according to the general procedure described in, e.g., Gharpure et al., J. Virol. 3:414-421 (1969) and Richardson et al., J. Med. Virol. 3:91-100 (1978). Other chemical mutagens can also be used. Attenuation can result from a ts mutation in almost any RS virus gene. The level of temperature sensitivity of the replication of the attenuated RS virus of the invention is determined by comparing its replication at a permissive temperature with that at several restrictive temperatures. The lowest temperature at which the replication of the virus is reduced 100-fold or more in comparison with its replication at the permissive temperature is termed the shutoff temperature. In experimental animals and humans, both the replication and virulence of RS virus correlate with the mutant's shutoff temperature. Replication of mutants with a shutoff temperature of 39°C is moderately restricted, whereas mutants with a shutoff of 38°C replicate less well and symptoms of illness are mainly restricted to the upper respiratory tract. A virus with a shutoff temperature of 35 to 37°C should be fully attenuated in humans. Thus, the attenuated RS virus of the invention which is temperature-sensitive will have a shutoff temperature in the range of about 35 to 39°C, and preferably from 35 to 38°C. The addition of the temperature sensitive property to a partially attenuated strain produces completely attenuated virus useful in the vaccine compositions of the present invention.

In addition to the criteria of viability, attenuation and immunogenicity, the properties of the derivative which are selected must also be as stable as possible so that the desired attributes are maintained. Genetic instability of the ts phenotype following replication in vivo has been the rule for ts viruses (Murphy et al., Infect. and Immun. 37:235-242 (1982)). Ideally, then, the virus which is useful in the vaccines of the present invention must maintain its viability, its property of attenuation, its ability to replicate in the immunized host (albeit at lower levels), and its ability to effectively elicit the production of an immune response in the vaccinee that is sufficient to confer protection against serious disease caused by subsequent infection by wild-type virus.

Clearly, the heretofore known and reported RS virus mutants do not meet all of these criteria. Indeed, contrary to expectations based on the results reported for known attenuated RS viruses, some of the viruses of the invention, which have a minimun of two to three distinct mutations, are not only viable and more attenuated then previous mutants, but are more stable genetically in vivo than those previously studied mutants, retaining the ability to stimulate a protective immune response and in some instances to expand the protection afforded by multiple modifications, e.g., induce protection against different viral strains or subgroups, or protection by a different immunologic basis, e.g., secretory versus serum immunoglobulins, cellular immunity, and the like.

Propagation of the attenuated virus of the invention may be in a number of cell lines which allow for RS virus growth. RS virus grows in a variety of human and animal cells. Preferred cell lines for propagation of attenuated RS virus for vaccine use include DBS-FRhL-2, MRC-5, and Vero cells. Highest virus yields are usually achieved with heteroploid cell lines such as Vero cells. Cells are typically inoculated with virus at a multiplicity of infection ranging from about 0.001 to 1.0 or more, and are cultivated under conditions permissive for replication of the virus, e.g., at about 30°-37°C and for about 3-5 days, or as long as necessary for virus to reach an adequate titer. Virus is removed from cell culture and separated from cellular components, typically by well known clarification procedures, e.g., centrifugation, and may be further purified as desired using procedures well known to those skilled in the art.

RS virus which has been attenuated as described herein can be tested in in vitro and in vivo models to confirm adequate attenuation, genetic stability, and immunogencity for vaccine use. In in vitro assays the modified virus is tested for the small plaque phenotype. Modified viruses are further tested in animal models of RS infection. A variety of animal models have been described and are summarized in Meignier et al., eds., Animal Models of Respiratory Syncytial Virus Infection, Merieux Foundation Publication, (1991), which is incorporated herein by reference. A cotton rat model of RS infection is described in U.S. 4,800,078 and Prince et al., Virus Res. 3:193-206 (1985), which are incorporated herein by reference, and is believed to be predictive of attenuation and efficacy in humans. A primate model of RS infection using the chimpanzee is predictive of attenuation and efficacy in humans, and is described in detail in Richardson et al., J. Med. Virol. 3:91-100 (1978); Wright et al., Infect. Immun., 37:397-400 (1982); Crowe et al., Vaccine (1993) (in press), which are incorporated herein by reference.

For example, the therapeutic effect of RSV neutralizing antibodies in infected cotton rats has been shown to be highly relevant to subsequent experience with

immunotherapy of monkeys and humans infected with RSV. Indeed, the cotton rat appears to be a reliable experimental surrogate for the response of infected monkeys and humans to immunotherapy with RSV neutralizing antibodies. For example, the amount of RSV neutralizing antibodies associated with a therapeutic effect in cotton rats as measured by the level of such antibodies in the serum of treated animals (i.e., serum RSV neutralization titer of 1:302 to 1:518) is in the same range as that demonstrated for monkeys (i.e., titer of 1:539) or human infants or small children (i.e., 1:877). A therapeutic effect in cotton rats was manifest by a one hundred fold or greater reduction in virus titer in the lung (Prince et al., J. Virol. 61:1851-1854) while in monkeys a therapeutic effect was observed to be a 50-fold reduction in pulmonary virus titer. (Hemming et al., J. Infect. Dis. 152:1083-1087 (1985)). Finally, a therapeutic effect in infants and young children who were hospitalized for serious RSV bronchiolitis or pneumonia was manifest by a significant increase in oxygenation in the treated group and a significant decrease in amount of RSV recoverable from the upper respiratory tract of treated patients. (Hemming et al., Antimicrob. Agents Chemother. 31:1882-1886 (1987)). Therefore, based on these studies, it would appear that the cotton rat constitutes a relevant model for predicting the success of an RSV vaccine in infants and small children. Other rodents, including hamsters and mice, should also be similarly useful because these animals are permissive for RSV replication and have a core temperature more like that of humans (Wight et al., J. Infect. Dis. 122:501-512 (1970) and Anderson et al., J. Gen. Virol. 71:(1990)).

For vaccine use, the attenuated virus of the invention can be used directly in vaccine formulations, or lyophilized, as desired, using lyophilization protocols well known to the artisan. Lyophilized virus will typically be maintained at about 4°C. When ready for use the lyophilized virus is reconstituted in a stabilizing solution, e.g., saline or comprising SPG, Mg^{++} and HEPES, with or without adjuvant, as further described below.

Thus RS virus vaccines of the invention contain as an active ingredient an immunogenetically effective amount of an attenuated RS virus as described herein. The attenuated virus may be introduced into a host, particularly humans, with a physiologically acceptable carrier and/or adjuvant. Useful carriers are well known in the art, and include, e.g., water, buffered water, 0.4% saline, 0.3% glycine, hyaluronic acid and the like. The resulting aqueous solutions may be packaged for use as is, or lyophilized, the lyophilized preparation being combined with a sterile solution prior to administration, as mentioned above. The compositions may contain pharmaceutically acceptable auxiliary substances as required to approximate

physiological conditions, such as pH adjusting and buffering agents, tonicity adjusting agents, wetting agents and the like, for example, sodium acetate, sodium lactate, sodium chloride, potassium chloride, calcium chloride, sorbitan monolaurate, triethanolamine oleate, and the like.

Upon inoculation with an attenuated RS virus composition as described herein, via aerosol, droplet, coarse spray, oral, topical or other route, most preferably suitable for intranasal delivery, the immune system of the host responds to the vaccine by producing antibodies, both secretory and serum, specific for RS virus proteins. As a result of the vaccination the host becomes at least partially or completely immune to RS virus infection, or resistant to developing moderate or severe RS viral infection, particularly of the lower respiratory tract.

The vaccine compositions containing the attenuated RS virus of the invention are administered to a person susceptible to or otherwise at risk of RS virus infection to enhance the individual's own immune response capabilities. Such an amount is defined to be a "immunogenically effective dose." In this use, the precise amounts again depend on the patient's state of health and weight, the mode of administration, the nature of the formulation, etc., but generally range from about 10^3 to about 10^6 plaque forming units (PFU) or more of virus per patient, more commonly from about 10^4 to 10^5 PFU virus per patient. In any event, the vaccine formulations should provide a quantity of attenuated RS virus of the invention sufficient to effectively protect the patient against serious or life-threatening RS virus infection.

The attenuated RS virus of the invention of one particular RS subgroup or strain can be combined with attenuated viruses of the other subgroup or strains to achieve protection against multiple RS viruses. Typically the different modified viruses will be in admixture and administered simultaneously, but may also be administered separately. Due to the phenomenon of cross-protection among certain strains of RS virus, immunization with one strain may protect against several different strains of the same or different subgroup.

In some instances it may be desirable to combine the attenuated RS virus vaccines of the invention with vaccines which induce protective responses to other agents, particularly other childhood viruses. For example, the attenuated virus vaccine of the present invention can be administered simultaneously (typically separately) or sequentially with parainfluenza virus vaccine, such as described in Clements et al., J. Clin Microbiol. 29:1175-1182 (1991), which is incorporated herein by reference.

Single or multiple administrations of the vaccine compositions of the invention can be carried out. In neonates and infants, multiple administration may be

required to elicit sufficient levels of immunity. Administration should begin within the first month of life, and continue at intervals throughout childhood, such as at two months, six months, one year and two years, as necessary to maintain sufficient levels of protection against native (wild-type) RS virus infection. Similarly, adults who are particularly susceptible to repeated or serious RS virus infection, such as, for example, health care workers, day care workers, family members of young children, elderly, individuals with compromised cardiopulmonary function, etc. may require multiple immunizations to establish and/or maintain protective imune responses. Levels of induced immunity can be monitored by measuring amounts of neutralizing secretory and serum antibodies, and dosages adjusted or vaccinations repeated as necessary to maintain desired levels of protection.

The following examples are provided by way of illustration, not limitation.

EXAMPLE I

Isolation and Characterization of Mutagenized Derivatives of Cold-passaged RSV

This Example describes the chemical mutagenesis of incompletely attenuated host range-restricted cpRSV to produce derivative ts and sp strains which are more highly attenuated and thus are preferred for use in RSV vaccine preparations.

A parent stock of cold-passaged RSV (cpRSV) was prepared. Flow Laboratories Lot 3131 virus, the cpRSV parent virus that is incompletely attenuated in humans, was passaged twice in MRC-5 cells at 25°C, terminally diluted twice in MRC-5 cells at 25°C, then passaged three times in MRC-5 to create a cpRSV suspension for mutagenesis.

The cpRSV was mutagenized by growing the parent stock in MRC-5 cells at 32°C in the presence of 5-fluorouracil in the medium at a concentration of 4 x 10^{-4}M. This concentration was demonstrated to be optimal in preliminary studies, as evidenced by a 100-fold decrease in virus titer on day 5 of growth in cell culture, compared to medium without 5-fluorouracil. The mutagenized stock was then analyzed by plaque assay on Vero cells that were maintained under an agar overlay, and after an appropriate interval of incubation, plaques were stained with neutral red dye. 854 plaques were picked and the progeny of each plaque were separately amplified by growth on fresh monolayers of Vero cells. The contents of each of the tissue cultures inoculated with the progeny of a single plaque of cpRSV-mutagenized virus were

separately harvested when cytopathic effects on the Vero cells appeared maximal. Progeny virus that exhibited the temperature-sensitive (ts) or small-plaque (sp) phenotype was sought by titering these plaque pools on HEp-2 cells at 32°C and 38°C. Any virus exhibiting a sp phenotype (plaque size that was reduced by 50% or more compared to parental virus at 32°C) or a ts phenotype (100-fold reduction in titer at restrictive temperature [37° to 40°C] compared to 32°C) was evaluated further. These strains were biologically cloned by serial plaque-purification on Vero cells three times, then amplified on Vero cells. The cloned strains were titered at 32°, 37°, 38°, 39° and 40°C (in an efficiency of plaque formation (EOP) assay) to confirm their sp and ts phenotypes. Because titers of some cloned strains were relatively low even at the permissive temperature (32°), these viruses were passaged once in HEp-2 cells to create virus suspensions for in vitro analysis. The phenotypes of the progeny of the mutagenized cpRSV are presented on Table 1.

Table 1 The efficiency of plaque formation of nine derivatives of cold-passaged RSV (*cpts* or *cpsp* mutants) in HEp-2 cells at permissive and restrictive temperatures

	The titer of virus (log_{10}pfu/ml) at the indicated temperature (°C)					Shut-off temperature	Small-plaques
Virus	32	37	38	39	40	(°C)[1]	at 32C
A2 wild-type	4.5	4.4	4.5	3.8	3.8	>40	no
cp-RSV	6.0	5.8	5.8	6.2	5.4	>40	no
ts-1	5.7	4.5	**2.7**	2.4	1.7*	38	no
cpsp-143	4.2*	4.1*	3.8*	3.9*	3.8*	>40	yes
cpts-368	6.7	6.3	6.1*	5.8**	**2.0****	40	no
cpts-274	7.3	7.1	6.6	5.8*	**1.0****	40	no
cpts-347	6.2	6.1	5.7*	5.5**	**<0.7**	40	no
cpts-142	5.7	5.1	4.5*	**3.7****	<0.7	39	no
cpts-299	6.2	5.5	5.1*	**2.0****	<0.7	39	no
cpts-475	5.4	4.8*	4.2**	**<0.7**	<0.7	39	no
cpts-530	5.5	4.8*	4.5*	**<0.7**	<0.7	39	no
cpts 248	6.3	5.3**	**<0.7**	<0.7	<0.7	38	no

[1]Shut-off temperature is defined as the lowest restrictive temperature at which a 100-fold or greater reduction of plaque titer is observed (**bold** figures in table).
*Small-plaque phenotype (<50% wild-type plaque size)
**Pinpoint-plaque phenotype (<10% wild-type plaque size)

One of the mutant progeny had the small plaque phenotype, RSV cpsp-143 (sp refers to the small plaque (sp) phenotype), and the remaining mutant progeny had the ts phenotype. The RSV cpts mutants exhibit a variation in ability to produce plaques in monolayer cultures in vitro over the temperature range 37°C to 40°C, with cpts 368 retaining the ability to produce plaques at 40°C, whereas the most temperature-sensitive (ts) virus, cpts 248, failed to produce plaques at 38°C. Thus, several of the mutagenized cpRSV progeny exhibit a marked difference from their cpRSV parent virus with respect to temperature-sensitivity of plaque formation.

Replication and Genetic Stability Studies In Mice

The level of replication of the cpRSV progeny virus in the upper and lower respiratory tracts of BALB/c mice was studied next (Table 2). It was found that cpts 530 and cpts 248, two of the most ts viruses (see Table 1), were about 7- to 12-fold restricted in replication in the nasal turbinates of the mice (Table 2). However, none of the viruses was restricted in replication in the lungs compared to the cpRSV parent virus. This greater restriction of replication in the nasal turbinates than in the lungs is not characteristic of ts mutants, which generally are more restricted in replication in the warmer lower respiratory tract (Richman and Murphy, Rev. Infect. Dis. 1:413-433 (1979). The virus produced in the lungs and nasal turbinates retained the ts character of the input virus (data not presented). The present findings suggested that the combination of the ts mutations on the background of the mutations of the cp parent virus has resulted in cpRSV ts progeny with a higher level of stability of the ts phenotype after replication in vivo than had been seen with previously studied ts mutants.

To further explore the level of genetic stability of the ts phenotype of the cpRSV progeny viruses, the efficiency of plaque formation of virus present in the lungs and nasal turbinates of nude mice was studied for two mutagenized cpRSV progeny that were among the most ts, namely ts 248 and ts 530. Nude mice were selected because they are immunocompromised due to congenital absence of functional T-cells, and a virus can replicate for a much longer period of time in these hosts. This longer period of replication favors the emergence of virus mutants with altered phenotype. The virus present on day 12 (NOTE: in normal mice, virus is no longer detectable at this time) was characterized and found to retain an unaltered ts phenotype (Table 3). As expected, the ts-1 mutant included in the test as a positive control exhibited an unstable

ts phenotype in vivo. Thus, contrary to previous evaluation of ts mutant viruses in rodents, the results show that a high level of stability of the ts phenotype following prolonged replication in rodents was achieved, which represents a significant and heretofore unattained very desirable property in the viruses of the invention.

Table 2 Replication of *cpts* - RSV mutants in BALB/c mice[1]

		Virus titer at 32°C (mean $\log_{10}$pfu/g tissue from the tissues of eight animals ± standard error)			
		Day 4		Day 5	
Animals infected with	Shutoff temperature of virus (°C)	Nasal Turbinates	Lungs	Nasal turbinates	Lungs
A2 wild-type	>40	5.0 ± 0.16	5.8 ± 0.20	5.0 ± 0.11	5.8 ± 0.19
cp-RSV	>40	4.7 ± 0.07	5.3 ± 0.18	4.8 ± 0.16	5.3 ± 0.21
ts-1	38	4.0 ± 0.19	4.7 ± 0.27	3.8 ± 0.33	4.9 ± 0.13
cpsp-143	>40	4.5 ± 0.14	4.1 ± 0.37	4.4 ± 0.39	4.6 ± 0.39
cpts-368	40	4.8 ± 0.15	5.1 ± 0.35	4.7 ± 0.08	5.4 ± 0.23
cpts-274	40	4.2 ± 0.19	5.0 ± 0.15	4.2 ± 0.11	5.1 ± 0.55
cpts-347	40	4.4 ± 0.32	4.9 ± 0.40	4.5 ± 0.33	5.2 ± 0.35
cpts-142	39	4.1 ± 0.34	5.0 ± 0.19	4.3 ± 0.24	5.8 ± 0.40
cpts-299	39	3.9 ± 0.11	5.2 ± 0.15	3.9 ± 0.32	5.0 ± 0.29
cpts-475	39	4.0 ± 0.18	5.3 ± 0.25	4.1 ± 0.23	4.9 ± 0.42
cpts-530	39	3.9 ± 0.18	5.3 ± 0.15	3.9 ± 0.14	5.3 ± 0.19
cpts-248	38	3.9 ± 0.33	5.1 ± 0.29	4.2 ± 0.13	5.5 ± 0.35

[1]Mice were administered $10^{6.3}$ p.f.u. intranasally in a 0.1 ml inoculum on day 0, then sacrificed on day 4 or 5.

Table 3 The genetic stability of RSV *cpts*-248 and *cpts*-530 following prolonged replication in nude mice

Efficiency of plaque formation at indicated temperature of virus present in nasal turbinates (n.t.) or lungs of nude mice sacrificed 12 days after virus administration[1]

			32°C		37°C			38°C			40°C		
Animals infected with	Tissue harvest or input virus tested	Number of animals	% animals with virus detectable	Mean titer (log_{10}pfu per gram tissue or ml inoculum)	% animals with virus detectable	% animals with virus with altered ts phenotype	Mean titer (log_{10}pfu per gram tissue or ml inoculum)	% animals with virus detectable	% animals with virus with altered ts phenotype	Mean titer (log_{10}pfu per gram tissue or ml inoculum)	% animals with virus detectable	%animals with virus with altered ts phenotype	Mean titer (log_{10}pfu per gram tissue or ml inoculum)
cpts-248	n.t.	19	100	3.8 ± 0.34	0	0	<2.0	0	0	<2.0	0	0	<2.0
"	lungs	"	90	2.0 ± 0.29	0	0	<1.7	0	0	<1.7	0	0	<1.7
cpts-530	n.t.	20	100	3.0± 0.26	0	0	<2.0	0	0	<2.0	0	0	<2.0
"	lungs	"	100	2.4 ± 0.29	0	0	<1.7	0	0	<1.7	0	0	<1.7
ts-1	n.t.	19	100	3.7 ± 0.23	74	74	2.7 ± 0.57	63	63	2.4 ± 0.36	10	10	2.0 ± 0.13
"	lungs	"	100	2.5 ± 0.30	74	74	1.8 ± 0.21	35	32	1.8 ± 0.15	0	0	<1.7
	cpts-248	-	-	4.9	-		<0.7	-		<0.7	-		<0.7
	cpts-530	-	-	5.5	-		3.7*	-		<0.7	-		<0.7
	ts-1	-	-	6.1	-		3.3	-		2.7	-		<0.7

[1] Plaque titers shown represent the mean log_{10}pfu/gram tissue of 19 or 20 samples ± standard error

[2] Each animal received $10^{6.3}$ p.f.u. intranasally in a 0.1 ml inoculum of the indicated virus on day 0.

*Small-plaque phenotype only.

virus possesses two highly desirable properties for a live RSV vaccine, namely, evidence of attenuation in both the upper and the lower respiratory tracts of highly susceptible seronegative chimpanzees. The level of genetic stability of the virus present in the respiratory tract of chimpanzees was evaluated next (Table 5). The virus present in the respiratory tract secretions retained the ts phenotype, and this was seen even with the virus from chimpanzee No. 3 on day 8 that was reduced 100-fold in titer at 40°C and exhibited the small plaque phenotype at 40°C, indicating that its replication was still temperature-sensitive. This represents the most genetically stable ts mutant identified to date. The increased stability of the ts phenotype of the cpts 248 and cpts 530 viruses reflects an effect of the cp mutations on the genetic stability of the mutations that contribute to the ts phenotype in vivo. Thus, the ts mutations in the context of the mutations present in the cp3131 parent virus appear to be more stable than would be expected in their absence. This important property has not been previously observed or reported. Infection of chimpanzees with the cpts 248 induced a high titer of neutralizing antibodies, as well as antibodies to the F and G glycoproteins (Table 6). Significantly, immunization with cpts 248 protected the animals from RSV challenge (Table 7), indicating that this mutant functions as an effective vaccine virus in a host that is closely related to humans.

These above-presented findings indicate that the cpts 248 virus has many properties desirable for a live RSV vaccine, including: 1) attenuation for the upper and lower respiratory tract; 2) increased genetic stability after replication in vivo, even after prolonged replication in immunosuppressed animals; 3) satisfactory immunogenicity; and 4) significant protective efficacy against challenge with wild-type RSV. The cpts 530 virus shares with cpts 248 similar temperature sensitivity of plaque formation, a similar degree of restriction of replication in the nasal turbinates of mice, and a high level of genetic stability in immunodeficient nude mice, whereby it also represents an RS virus vaccine strain.

Table 4 Replication of *cpts*-RSV 248, *cp*-RSV, or wild-type RSV A2 in the upper and lower respiratory tract of seronegative chimpanzees

			Virus recovery					
			Nasopharynx		Trachea		Rhinorrhea score	
Animal infected with indicated virus	Route of Inoculation	Chimpanzee number	Duration[b] (days)	Peak titer (log_{10}pfu/ml)	Duration[b] (days)	Peak titer (log_{10}pfu/ml)	Mean[c]	Peak
cpts-248	IN + IT	1	10	4.6	8[d]	5.4	0.2	1
	IN + IT	2	10	4.5	6	2.2	0.1	1
	IN + IT	3	9	4.7	10	2.1	0.1	1
	IN + IT	4	9	4.2	8[d]	2.2	0.1	1
			mean 9.5	mean 4.5	mean 8.0	mean 3.0	mean 0.1	
cp-RSV	IN	5	20	5.3	8[d]	2.9	1.0	3
	IN	6	16	5.8	6[d]	3.0	1.8	3
	IN + IT	7	13	4.3	6[d]	3.0	0.6	1
	IN + IT	8	16	5.0	10[d]	2.8	0.5	1
			mean 16	mean 5.1	mean 7.5	mean 2.9	mean 1.0	
A2 wild-type	IN	9	9	5.1	13	5.4	1.0	1
	IN	10	9	6.0	8	6.0	1.7	4
	IN + IT	11	13	5.3	8	5.9	2.1	3
	IN + IT	12	9	5.4	8	5.6	1.0	3
			mean 10	mean 5.5	mean 9.3	mean 5.7	mean 1.4	

[a]IN = Intranasal administration only, at a dose of 10^4 p.f.u. in a 1.0 ml inoculum; IN + IT = Both intranasal and intratracheal administration, 10^4 p.f.u. in a 1.0 ml inoculum at each site.

[b]Indicates last day post-infection on which virus was recovered.

[c]Mean rhinorrhea score represents the sum of daily scores for a period of eight days surrounding the peak day of virus shedding, divided by eight. Four is the highest score; zero is the lowest score

[d]Virus isolated only on day indicated.

Table 5 Genetic stability of virus present in original nasopharyngeal (NP) swabs or tracheal lavage (TL) specimens obtained from animals experimentally infected with *cpts*-RSV 248

Chimpanzee number	NP swab or TL specimen	Virus obtained on post-infection day	Titer of RSV at indicated temperature (log_{10}pfu/ml) Titer at 32°C	Titer at 39°C	Titer at 40°C
1[a]	NP	3	3.2	<0.7	NT
	"	4	2.7	<0.7	NT
	"	5	4.2	<0.7	NT
	"	6	3.8	<0.7	NT
	"	7	4.6	<0.7	NT
	"	8	4.5	<0.7	NT
	"	9	2.6	<0.7	NT
	"	10	2.0	<0.7	NT
	TL	6	5.4	<0.7	NT
	"	8	2.7	<0.7	NT
2[a]	NP	3	3.2	<0.7	NT
	"	4	3.7	<0.7	NT
	"	5	4.5	<0.7	NT
	"	6	4.1	<0.7	NT
	"	7	3.3	<0.7	NT
	"	8	4.2	<0.7	NT
	"	9	2.8	<0.7	NT
	"	10	1.6	<0.7	NT
	TL	6	2.2	<0.7	NT
3	NP	3	2.7	<0.7	<0.7
	"	4	3.4	<0.7	<0.7
	"	5	2.9	<0.7	<0.7
	"	6	3.3	<0.7	<0.7
	"	7	3.4	0.7[b]	<0.7
	"	8	4.7	3.5[b]	2.0[c]
	"	9	1.9	<0.7	<0.7
	TL	6	1.8	<0.7	<0.7
	"	8	1.9	1.2[b]	<0.7
	"	10	2.1	1.3[b]	<0.7
4	NP	3	3.2	<0.7	NT
	"	4	2.7	<0.7	NT
	"	5	3.4	<0.7	NT
	"	6	3.3	<0.7	NT
	"	7	4.2	<0.7	NT
	"	8	3.5	<0.7	NT
	"	9	2.1	<0.7	NT
	TL	8	2.2	<0.7	NT

NT=Not tested

[a]Isolates (once-passaged virus suspensions with average titer log_{10}pfu/ml of 4.0) were generated for samples from these chimpanzees from each original virus-containing nasopharyngeal swab specimen or tracheal lavage specimen and tested for efficiency of plaque formation at 32°, 39° and 40°C. No isolate was able to form plaques at 39°C or 40°C. Isolates from chimpanzees 3 and 4 were not tested in this manner.

[b]The percent titer at 39°C versus that at 32°C: NP swab day 7 = 0.2%, NP swab day 8 = 6%. TL day day 8 = 20%, TL day 10 = 16%. All plaques were of small-plaque phenotype only; no wild-type size plaques seen.

[c]The percent titer at 40°C versus that at 32°C was 0.2%. All plaques were of pinpoint-plaque phenotype; wild-type size plaques were not detected.

Table 6 Serum antibody responses of chimpanzees infected with RSV *cpts*-248, *cp*-RSV, or RSV A2 wild-type

Animals immunized with	Number of chimpanzees	Serum antibody titers (reciprocal mean log_2)					
		Neutralizing		ELISA-F		ELISA-G	
		Day 0	Day 28	Day 0	Day 28	Day 0	Day 28
cpts-248	4	<3.3	10.7	7.3	15.3	6.3	9.8
cp-RSV	4	<3.3	11.2	11.3	15.3	9.3	12.3
RSVA2 wild-type	4	<3.3	11.2	8.3	15.3	7.3	10.3

Table 7 Immunization of chimpanzees with *cpts*-248 induces resistance to RSV A2 wild-type virus challenge on day 28

		Response to challenge with 10^4 p.f.u. wild-type virus administered on day 28							
		Virus Recovery				Rhinorrhea score		Serum neutralizing antibody titer (reciprocal $\log_2$) on day indicated	
		Nasopharynx		Trachea					
Virus used to immunize animal	Chimpanzee number	Duration (days)	Peak titer ($\log_{10}$pfu/ml)	Duration (days)	Peak titer ($\log_{10}$pfu/ml)	Mean[a]	Peak	Day 28	Day 42 or 56
cpts-248	1	5	2.7	0	<0.7	0	0	10.1	11.0
	2	9	1.8	0	<0.7	0	0	10.3	14.5
cp-RSV	5	5	1.0	0	<0.7	0	0	11.1	13.3
	6	8	0.7	0	<0.7	0	0	11.4	12.9
none	9	9	5.1	13	5.4	1.0	1	<3.3	12.4
	10	9	6.0	8	6.0	1.7	4	<3.3	13.2
	11	13	5.3	8	5.9	2.1	3	<3.3	11.6
	12	9	5.4	8	5.6	1.0	3	<3.3	11.2

[a]Mean rhinorrhea score represents the sum of scores during the eight days of peak virus shedding divided by eight. Four is the highest score. A score of zero indicates no rhinorhea detected on any day of the ten-day observation period.

Further Attenuations

Since RS virus produces more symptoms of lower respiratory tract disease in humans then in chimpanzees, and recognizing that mutants which are satisfactorily attenuated for the chimpanzee may not be so for seronegative infants and children, the cpts 248 and 530 derivatives, which possess the very uncharacteristic ts mutant properties of restricted replication and attenuation in the upper respiratory tract and a higher level of genetic stability, were further mutagenized.

Progeny viruses that exhibited a greater degree of temperature-sensitivity in vitro than cpts 248 or that had the small plaque phenotype were selected for further study. Mutant derivatives of the cpts 248 that possessed one or more additional ts mutations were produced by 5-fluorouracil mutagenesis (Table 8). Ts mutants that were more temperature-sensitive (ts) than the cpts 248 were identified, and some of these had the small plaque (sp) phenotype. These cpts 248 derivatives were administered to mice. Cpts 248/804, 248/955, 248/404, 248/26, 248/18, and 248/240 mutants were more restricted in replication in the upper and lower respiratory tract of the mouse than their cpts 248 parental virus (Table 9). Thus, viable mutants of cpts 248 which were more attenuated than their cpts 248 were identified, and these derivatives of cpts 248 exhibited a wide range of replicative efficiency in mice, with ts 248/26 being the most restricted. The ts phenotype of the virus present in nasal turbinates and lungs of the mice was almost identical to that of the input virus, indicating genetic stability. A highly attenuated derivative of cpts 248, the cpts 248/404 virus, was 1000-fold more restructed in replication in the nasopharynx compared to wild-type. The cpts 248/404 mutant, possessing at least three attenuating mutations, was also highly restricted in replication in the upper and lower respiratory tracts of two seronegative chimpanzees and infection did not induce rhinorrhea (Table 10). Again, this virus exhibited a high degree of reduction in replication compared to wild-type, being 60,000-fold reduced in the nasopharynx and 100,000-fold in the lungs. Nonetheless, these two chimpanzees were highly resistant to subsequent challenge with RSV wild-type virus (Table 11). In addition, further ts derivatives of the cpts 530 virus were also generated (Table 12). These results represent yet a further improvement in the properties of the RS viruses of the invention and also represent a very important and significant advance in the development of RS virus vaccine strains.

These results were completely unexpected based on experience gained during prior studies. For example, the results of an earlier study indicated that the in vivo properties of RSV ts mutants derived from a single cycle of 5-fluorouracil

mutagenesis could not be predicted a priori. Moreover, although one of the first four ts mutants generated in this manner exhibited the same shut off temperature for plaque formation as the other mutants, it was overattenuated when tested in susceptible chimpanzees and susceptible infants and young children [Wright et al., Infect Immun. 37 (1):397-400 (1982)]. This indicated that the acquisition of the ts phenotype resulting in a 37°-38°C shut off temperature for plaque formation did not reliably yield a mutant with the desired level of attenuation for susceptible chimpanzees, infants and children. Indeed, the results of studies with heretofore known ts mutants completely fail to provide any basis for concluding that introduction of three independent mutations (or sets of mutations) into RSV by cold-passage followed by two successive cycles of chemical mutagenesis could yield viable mutants which retain infectivity for chimpanzees (and by extrapolation, young infants) and exhibit the desired level of attenuation, immunogenicity and protective efficacy required of a live virus vaccine to be used for prevention of RSV disease.

The above-presented results clearly demonstrate that certain ts derivatives of the cpRSV of the invention are infectious and exhibit a significant degree of attenuation for mice and chimpanzees. These ts mutant derivatives are attenuated and appear highly stable genetically after replication in vivo. These mutants also induce significant resistance to RSV infection in chimpanzees. Thus, these cpRSV derivatives represent virus strains suitable for use in a live RSV vaccine designed to prevent serious human RSV disease.

Table 8 The efficiency of plaque formation of ten mutants derived from RSV *cpts*-248 by additional 5FU mutagenesis

Virus	The titer of virus (log_{10}pfu/ml) at the indicated temperature (°C)							Shut-off temperature (°C)[1]	Small-plaques at 32C
	32	35	36	37	38	39	40		
A2 wild-type	4.5	4.6	4.4	4.5	4.5	3.8	3.8	>40	no
cp-RSV	4.7	4.4	4.3	4.3	4.2	3.7	3.5	>40	no
ts-1	5.6	5.4	4.9	4.4	**2.7**	2.0	<0.7	38	no
cpts-248	3.4	3.0	2.6*	1.7**	**<0.7**	<0.7	<0.7	38	no
248/ 1228	5.5*	5.3*	5.3**	**<0.7**	<0.7	<0.7	<0.7	37	yes
248/ 1075	5.3*	5.3*	5.1**	**<0.7**	<0.7	<0.7	<0.7	37	yes
248/ 965	4.5	4.2	4.2*	**<0.7**	<0.7	<0.7	<0.7	37	no
248/967	4.4	3.7	3.6*	**<0.7**	<0.7	<0.7	<0.7	37	no
248/ 804	4.9	4.5	4.0*	**<0.7**	<0.7	<0.7	<0.7	37	no
248/ 955	4.8	3.7	**2.8***	<0.7	<0.7	<0.7	<0.7	36	no
248/ 404	3.6	2.9*	**<0.7**	<0.7	<0.7	<0.7	<0.7	36	no
248/ 26	3.1	2.9*	**<0.7**	<0.7	<0.7	<0.7	<0.7	36	no
248/ 18	4.0*	4.0**	**<0.7**	<0.7	<0.7	<0.7	<0.7	36	yes
248/ 240	5.8*	5.7**	**<0.7**	<0.7	<0.7	<0.7	<0.7	36	yes

[1]Shut-off temperature is defined as the lowest restrictive temperature at which a 100-fold or greater reduction of plaque titer in HEp-2 cells is observed (**bold** figures in table).

*Small-plaque phenotype (<50% wild-type plaque size)

**Pinpoint-plaque phenotype (<10% wild-type plaque size)

Table 9 Replication and genetic stability of ten mutants derived from RSV*cpts*-248 in Balb/c mice[1]

Virus used to infect animal	Shutoff temperature of virus (°C)	Virus titer (mean log_{10}pfu/g tissue of six animals ± standard error)							
		Nasal turbinates				Lungs			
		32°C	36°C	37°C	38°C	32°C	36°C	37°C	38°C
A2 wild-type	>40	5.1 ± 0.15	5.2 ± 0.23	5.2 ± 0.14	5.2 ± 0.27	6.1 ± 0.14	5.8 ± 0.23	6.0 ± 0.12	5.9 ± 0.17
cp-RSV	>40	4.9 ± 0.20	5.1 ± 0.16	4.9 ± 0.24	4.9 ± 0.22	6.0 ± 0.16	5.9 ± 0.23	5.6 ± 0.15	5.6 ± 0.13
ts-1	38	3.9 ± 0.25	2.7 ± 0.27	2.4 ± 0.42	2.5 ± 0.29	4.1 ± 0.21	3.5 ± 0.23	2.6 ± 0.18	2.0 ± 0.23
cpts-248	38	4.0 ± 0.16	2.5 ± 0.34	<2.0	<2.0	4.4 ± 0.37	1.8 ± 0.15	<1.7	<1.7
248/1228	37	4.1 ± 0.15	2.4 ± 0.48	<2.0	<2.0	2.0 ± 0.37	<1.7	<1.7	<1.7
248/1075	37	4.2 ±0.18	2.4 ± 0.40	<2.0	<2.0	5.5 ± 0.16	3.5 ± 0.18	<1.7	<1.7
248/ 965	37	3.8 ± 0.23	<2.0	<2.0	<2.0	4.5 ± 0.21	3.4 ± 0.16	<1.7	<1.7
248/ 967	37	4.4 ± 0.20	<2.0	<2.0	<2.0	5.4 ± 0.20	3.6 ± 0.19	<1.7	<1.7
248/ 804	37	2.9 ± 0.19	<2.0	<2.0	<2.0	3.6 ± 0.19	<1.7	<1.7	<1.7
248/ 955	36	3.2 ± 0.10	<2.0	<2.0	<2.0	3.2 ± 0.22	<1.7	<1.7	<1.7
248/ 404	36	2.1 ± 0.31	<2.0	<2.0	<2.0	4.4 ± 0.12	1.8 ± 0.20	<1.7	<1.7
248/ 26	36	<2.0	<2.0	<2.0	<2.0	2.3 ± 0.20	<1.7	<1.7	<1.7
248/ 18	36	2.9 ± 0.99	<2.0	<2.0	<2.0	4.3 ± 0.23	1.8 ± 0.15	<1.7	<1.7
248/ 240	36	2.9 ± 0.82	<2.0	<2.0	<2.0	3.9 ± 0.12	<1.7	<1.7	<1.7

[1]Mice were administered $10^{6.3}$ p.f.u. intranasally under light anesthesia on day 0, then sacrificed by CO_2 asphyxiation on day 4.

Table 10 Replication of *cpts*-RSV 248/404, *cpts* 248/18, *cpts*-RSV 248, *cp*-RSV, or wild-type RSV A2 in the upper and lower respiratory tract of seronegative chimpanzees

			Virus recovery					
			Nasopharynx		Trachea		Rhinorrhea scores	
Animal infected with indicated virus	Route of Inoculation	Chimpanzee number	Duration[b] (days)	Peak titer (log_{10}pfu/ml)	Duration[b] (days)	Peak titer (log_{10}pfu/ml)	Mean[c]	Peak
cpts-248/404	IN + IT	13	0	<0.7	0	<0.7	0	0
	IN + IT	14	0	<0.7	0	<0.7	0	0
cpts-248#	IN + IT	1	10	4.6	8[d]	5.4	0.2	1
	IN + IT	2	10	4.5	6	2.2	0.1	1
	IN + IT	3	9	4.7	10	2.1	0.1	1
	IN + IT	4	9	4.2	8[d]	2.2	0.1	1
			mean 9.5	mean 4.5	mean 8.0	mean 3.0	mean 0.1	mean 1.0
cp-RSV#	IN	5	20	5.3	8[d]	2.9	1.0	3
	IN	6	16	5.8	6[d]	3.0	1.8	3
	IN + IT	7	13	4.3	6[d]	3.0	0.6	1
	IN + IT	8	16	5.0	10[d]	2.8	0.5	1
			mean 16	mean 5.1	mean 7.5	mean 2.9	mean 1.0	mean 2.0
A2 wild-type#	IN	9	9	5.1	13	5.4	1.0	1
	IN	10	9	6.0	8	6.0	1.7	4
	IN + IT	11	13	5.3	8	5.9	2.1	3
	IN +IT	12	9	5.4	8	5.6	1.0	3
			mean 10	mean 5.5	mean 9.3	mean 5.7	mean 1.4	mean 2.8

[a]IN = Intranasal only; IN + IT = Both intranasal and intratracheal administration.
[b]Indicates last day post-infection on which virus was recovered.
[c]Mean rhinorrhea score represents the sum of daily scores for a period of eight days surrounding the peak day of virus shedding, divided by eight. Four is the highest score; zero is the lowest score.
[d]Virus isolated only on day indicated.
*Not tested yet.
#These are the same animals included in Tables 4 and 7.

TABLE 11 Immunization of chimpanzees with *cpts*-248/404 induces resistance to RSV A2 wild-type virus challenge on day 28

Virus used to immunize animal	Chimpanzee number	Virus Recovery: Nasopharynx Duration (days)	Virus Recovery: Nasopharynx Peak titer (log_{10}pfu/ml)	Virus Recovery: Tracheal lavage Duration (days)	Virus Recovery: Tracheal lavage Peak titer (log_{10}pfu/ml)	Rhinorrhea scores: Mean[a]	Rhinorrhea scores: Peak	Serum neutralizing antibody titer (reciprocal log_2) on day indicated: Day 28	Serum neutralizing antibody titer (reciprocal log_2) on day indicated: Day 42 or 56
cpts-248/404	13	0	<0.7	0	<0.7	0	0	NYT	NYT
	14	8	3.4	0	<0.7	0	0	NYT	NYT
		mean 4.0	mean 2.0	mean 0	mean <0.7	mean 0	mean 0		
cp-ts-248#	1	5	2.7	0	<0.7	0	0	10.1	11.0
	2	9	1.8	0	<0.7	0	0	10.3	14.5
		mean 7.0	mean 2.3	mean 0	mean <0.7	mean 0	mean 0	mean 10.2	mean 12.8
cp-RSV#	5	5	1.0	0	<0.7	0	0	11.1	13.3
	6	8	0.7	0	<0.7	0	0	11.4	12.9
		mean 6.5	mean 0.9	mean 0	mean <0.7	mean 0	mean 0	mean 11.2	mean 13.1
None #	9	9	5.1	13	5.4	1.0	1	<3.3	12.4
	10	9	6.0	8	6.0	1.7	4	<3.3	13.2
	11	13	5.3	8	5.9	2.1	3	<3.3	11.6
	12	9	5.4	8	5.6	1.0	3	<3.3	11.2
		mean 10	mean 5.5	mean 9.2	mean 5.7	mean 1.4	mean 2.8	mean <3.3	mean 12.1

[a]Mean rhinorrhea score represents the sum of scores during the eight days of peak virus sheeding divided by eight. Four is the highest score.

NYT = not yet tested

#These are the same animals included in Tables 4, 7, and 10.

Table 12 The efficiency of plaque formation of 14 mutants derived from RSV *cpts*-530 and tested in HEp-2 cells at permissive and restrictive temperatures, compared with controls

		The titer of virus (log_{10}pfu/ml) at the indicated temperature (°C)				Shut-off temperature	Small-plaques
	Virus	32	35	36	37	(°C)[1]	at 32C
	cp-RSV	6.3	6.3	6.0	6.3	>40	no
	cpts-530	2.7	2.5	2.4*	2.2**	39	no
EXP 1	530/ 9	4.4	3.5*	3.0**	**<1.0**	37	no
	530/346	3.1	3.0*	2.3**	**<1.0**	37	no
	530/ 653	2.0	2.5*	**<1.0**	<1.0	36	no
	530/ 667	4.8	4.2*	**<1.0**	<1.0	36	no
	530/ 403	2.6*	**<1.0**	<1.0	<1.0	35	no
	530/ 188	3.2*	**<1.0**	<1.0	<1.0	35	yes
	530/ 464	3.6*	**<1.0**	<1.0	<1.0	35	yes
EXP 2	530/ 1009	4.4	4.4	3.0**	**<0.7**	37	no
	530/ 1178	4.1*	3.8*	2.5**	**<0.7**	37	yes
	530/ 1074	4.1	4.1*	2.4**	**<0.7**	37	no
	530/ 963	4.5	4.3*	**2.3***	<0.7	36	no
	530/977	4.6	4.2*	**0.7***	<0.7	36	no
	530/ 1030	3.2*	1.3**	**<0.7**	<0.7	36	yes
	530/ 1003	3.6	3.0*	**<0.7**	<0.7	36	no

*Small plaque phenotype (<50% wild-type plaque size)
**Pinpoint-plaque phenotype (<10% wild-type plaque size)
[1]Shut-off temperature is defined as the lowest restrictive temperature at which a 100-fold or greater reduction of plaque titer is observed (bold figures in table). For viruses that have a titer at 32C of ≤ 2.9, the shut-off temperature is defined as the lowest temperature at which the virus fails to produce plaques.

EXAMPLE II

Use of Cold Adaptation to Attenuate cpRSV Mutants

This Example describes the introduction of growth restriction mutations into incompletely attenuated host range-restricted cpRSV strains by further passage of the strains at increasingly reduced temperatures to produce derivative strains which are more satisfactorily attenuated for use in human vaccines.

These cold-adaptation (ca) approaches were used to introduce further attenuation into the cpRSV 3131 virus, which is incompletely attenuated in seronegative children.

Under the first strategy, a parent stock of cold-passaged RSV (cpRSV 3131) obtained from Flow Laboratories was prepared by passage in MRC-5 cells at 25°C as described in Example I. Briefly, cold-passaged virus was inoculated into MRC-5 or Vero cell monolayer culture at a multiplicity of infection of ≤ 0.01 and the infected cells were incubated for 3 to 14 days before subsequent passage. Virus was passaged over 20 times at 20-22°C to derive more attenuated virus. The technique of rapid passage, as soon as the first evidence of virus replication is evident (i.e., 3 to 5 days), was preferable for selection of mutants able to replicate efficiently at low temperatures. Additionally, an RSV subgroup B strain, St. Louis/14617/85 clone 1A1, was isolated in primary African Green monkey kidney cells, passaged and cloned in MRC cells (1A1-MRC14), and cold-passaged 51 times in these cells at 32 to 22°C.

A second strategy employed a biologically cloned derivative of the uncloned parental cpRSV 3131 virus. This virus was biologically cloned in bovine embryonic kidney (BEK) cells (the tissue used to originally derive the cpRSV 3131 virus--see Friedewald et al., J. Amer. Med. Assoc. 204:690-694 (1968). This cloned virus was then passaged at 10 day intervals in Vero cells at low temperature. Alternatively, the cpRSV 3131 virus was cloned by terminal dilution (TD2P4) in MRC-5 cells and passaged at 10-day intervals in Vero cells.

The third strategy involved selection of mutants that produce large plaques at low temperature. An RSV cp3131 derivative virus designated plaque D1 that produces large plaques at 25°C has been identified. This virus was derived from the third passage (P3) level of the cp3131-1 (BEK) lineage cp3131-17 (BEK) lineage. The largest plaque produced by P3 virus was amplified at 32°C, then re-plaqued at 25°C. Once again the largest plaque was selected, amplified, and re-plaqued. After five such

cycles, large plaque mutant virus D1 was obtained. D1 was biologically cloned by two additional cycles of plaque-to-plaque purification at 25°C.

Biologically cloned virus D1 produces distinctly and uniformly larger plaques at 25°C than cp3131 or wild type virus A2. Thus D1 is cold adapted by the criterion of large plaque size at 25°C. Preliminary studies have suggested that D1 is not temperature sensitive. At 37°C, D1 plaques are indistinguishable from those of wild-type RSV or cp3131, suggesting that D1 is not restricted in growth at this temperature. Consistent with this, D1 produces extensive cytopathic effects in Vero cell monolayers at 37°C and 40°C (i.e. the highest temperatures tested).

EXAMPLE III

Introduction of Further Attenuating Mutations into ts-RSV

This Example describes the use of ts mutants as parental viruses to produce more completely attenuated strains. Two RSV A2 ts mutants were selected for this process, namely ts-4 and ts-1 NG1. Two distinct methods were chosen to introduce additional mutations into the RSV ts mutants. First, the incompletely attenuated RSV ts mutant was subjected to chemical mutagenesis, and mutagenized progeny that are more temperature-sensitive with regard to plaque formation were selected for further analysis. Second, the RSV ts mutants were passaged at low temperature to select RSV ts mutants with the ca phenotype, i.e., increased capacity to replicate at suboptimal temperature compared to wild-type parental virus.

A parent stock of ts-1 NG1 virus was prepared from Flow Laboratories Lot M2 of live Respiratory Syncytial Virus (A-2) ts-1 NG-1 mutant, MRC-5 grown virus. This mutant, derived from the ts-1 mutant by a second round of 5-fluorouracil mutagenesis, possesses two or more independent ts mutations, but still induces substantial rhinorrhea in susceptible chimpanzees. This virus was passaged twice in Vero cells at 32°C to create a ts-1 NG-1 suspension for mutagenesis. The virus was then grown in the presence of $4x10^{-4}M$ 5-fluorouracil to induce mutations during replication or was exposed to 5-azacytidine at 36°C after 5-fluorouracil treatment. The mutagenized stock was then analyzed by plaque assay on Vero cells that were maintained under an agar overlay, and, after an appropriate interval of incubation, plaques were identified microscopically. 586 plaques were picked, and the progeny of each plaque were separately amplified by growth on fresh monolayers of Vero cells. The contents of each of the tissue cultures inoculated with the progeny of a single

plaque of mutagenized ts-1 NG-1 virus were separately harvested when cytopathic effects on the Vero cells appeared maximal. Progeny virus that was more temperature-sensitive than ts-1 NG1 was sought by titering these plaque pools on HEp-2 cells at 32°C and 36°C. Any virus exhibiting greater temperature sensitivity than ts-1 NG1 (i.e., 100-fold reduction in titer at restrictive temperature [36°C] compared to 32°C) was evaluated further. Six plaque progeny more ts than the RSV ts-1 NG-1 parent virus were identified and these strains were biologically cloned by serial plaque-purification on Vero cells three times, then amplified on Vero cells. The cloned strains were titered at 32°C, 35°C, 36°C, 37°C, and 38°C (efficiency of plaque formation assay) to confirm their ts phenotypes. Efficiency of plaque formation data generated by assay on HEp-2 cells further confirmed the phenotypes of the six mutants (Table 13).

The two most ts viruses, A-20-4 and A-37-8, were highly attenuated in mice compared to their ts-1 NG1 parent virus, indicating that acquisition of increased level of temperature sensitivity was accompanied by augmented attenuation (Table 14). These viruses were infectious for mice because they induced an antibody response. The ts-1 NG1/A-20-4 virus is attenuated for chimpanzees (Table 15) and infection of chimpanzees with ts-1 NG1/A-20-4 induced resistance to wild-type virus challenge (Table 16). Significantly, rhinorrhea does not occur.

Mutagenesis of the ts-4 virus was also performed, using the same method as for mutagenesis of ts-1 NG1, virus. Five plaque progeny that were more ts than the RSV ts-4 parent virus were identified (Table 17).

Also, mutations have been introduced into the ts-1 NG1 virus and into the ts-4 viruses by cold-passage. The ts-4 virus replicates to high titer at 22°C after 38 cold-passages.

Table 13 Efficacy of plaque formation of *ts*-1 NG1 derivatives

Virus	Titer (log_{10}pfu/ml) at indicated temperature				
	32°	35°	36°	37°	38°
A-20-4(4 - 1)[a]	5.9*	<1	<1	<1	<1
A-37-8(1 - 2)[a]	6.3	6.3	<1	<1	<1
A-15-7	3.5	ND	2.1	1.5	<1
A-25-8	5.3	ND	5.0*	4.8*	<1
A-21	5.1**	ND	4.8**	4.5**	<1
Ts1NG1	6.6	6.6	6.5	6.6	<1

a. 3x plaque purified
*Small-plaque phenotype (<50% wild-type plaque size)
**Pinpoint-plaque phenotype (<10% wild-type plaque size)

Table 14 Replication of *ts*-1 NG1 parent and progeny viruses in Balb/C mice

Virus	Dose (log 10)	Day Post-Infection	Titers in lung		Titers in nose	
			32°	38°	32°	38°
A2 wt	6.1	4	4.66±0.32[a]	4.80±0.16	3.18±0.40	3.29 ±0.33
		5	5.18±0.33	5.25±0.23	3.40±0.20	3.47±0.14
TsINGI	5.8	4	4.31±0.17	<2.0	2.82±0.25	<2.0
		5	3.98±0.12	<2.0	2.74±0.31	<2.0
A-20-4	6.1	4	<2.0	<2.0	<2.0	<2.0
		5	<2.0	<2.0	<2.0	<2.0
A-37-8	6.3	4	<2.0	<2.0	<2.0	<2.0
		5	<2.0	<2.0	<2.0	<2.0

a. Mean log 10±standard error. 6 animals/group.

Table 15 Replication of *ts*-1 NG1/A-20-4, *ts*-1 NG1, *ts*-1 or wild-type RSV A2 in the upper and lower respiratory tract of seronegative chimpanzees

			Virus recovery				Rhinorrhea score	
			Nasopharynx		Trachea			
Animal infected with indicated virus	Route of Inoculation	Chimpanzee number	Duration[b] (days)	Peak titer (log_{10}pfu/ml)	Duration[b] (days)	Peak titer (log_{10}pfu/ml)	Mean[c]	Peak
ts-1NG1/A-20-4	IN + IT	15	0	<0.7	0	<0.7	0	0
	IN + IT	16	0	<0.7	0	<0.7	0	0
	IN + IT	17	0	<0.7	0	<0.7	0	0
	IN + IT	18	16[d]	2.7	0	<0.7	0	0
			mean 4.0	mean 1.2	mean 0	mean <0.7	mean 0	mean 0
ts-1 NG1	IN	19[e]	8	4.2	0	<1.1	0.6	1
	IN	20[e]	7	3.9	0	<1.1	0.7	1
	IN	21[e]	13	5.4	0	<1.1	0.4	1
	IN	22[e]	10	5.2	10[d]	3.7[d]	0.6	2
			mean 9.5	mean 4.7	mean 0.25	mean 1.8	mean 0.6	mean 1.3
ts-1	IN	23[e]	16	3.4	0	<1.1	0.4	1
	IN	24[e]	13	4.4	0	<1.1	1.0	3
	IN	25[e]	13	5.0	13[d]	2.2	2.0	4
	IN	26[e]	10	3.4	0	<1.1	1.0	2
			mean 13	mean 4.1	mean 0.25	mean 1.4	mean 1.1	mean 2.5
A2 wild-type	IN	9[f]	9	5.1	13	5.4	1.0	1
	IN	10[f]	9	6.0	8	6.0	1.7	4
	IN + IT	11[e]	13	5.3	8	5.9	2.1	3
	IN + IT	12[e]	9	5.4	8	5.6	1.0	3
			mean 10	mean 5.5	mean 9.3	mean 5.7	mean 1.4	mean 2.8

[a]IN=Intranasal only; IN + IT = Both intranasal and intratracheal administration.

[b]Indicates last day post-infection on which virus was recovered.

[c]Mean rhinorrhea score represents the sum of daily scores for a period of eight days surrounding the peak day of virus shedding, divided by eight. Four is the highest score; zero is the lowest score.

[d]Virus isolated only on day indicated.

[e]Animals from Crowe, et al., Vaccine in press (1993).

[f]Animals from Collins, et al., Vaccine 8:164-168 (1990).

TABLE 16 Immunization of chimpanzees with 10^4 pfu of RSV *ts*-1 NG1/A-20-4, *ts*-1 NG1, or ts-1 induces resistance to 10^4 pfu RSV A2 wild-type virus challenge on day 28

Virus used to immunize animal	Chimpanzee number	Virus Recovery: Nasopharynx Duration (days)	Virus Recovery: Nasopharynx Peak titer (log_{10}pfu/ml)	Virus Recovery: Tracheal lavage Duration (days)	Virus Recovery: Tracheal lavage Peak titer (log_{10}pfu/ml)	Rhinorrhea scores: Mean[a]	Rhinorrhea scores: Peak	Serum neutralizing antibody titer (reciprocal log_2) on day indicated: Day 28	Serum neutralizing antibody titer (reciprocal log_2) on day indicated: Day 42 or 56
ts-1 NG1/A-20-4	15	0	<0.7	0	<0.7	0	0	<3.3	Pending
	16	0	<0.7	0	<0.7	0	0	<3.3	Pending
	17	0	<0.7	0	<0.7	0	0	6.8	Pending
	18	3	2.0	0	<0.7	0	0	9.7	Pending
		mean 0.8	mean 1.0	mean 0	mean <0.7	mean 0	mean 0	mean 5.8	mean
ts-1 NG1	19[b]	0	<0.7	0	<1.1	0	0	11.4	10.4
	20[b]	0	<0.7	0	<1.1	0	0	14.4	12.4
	21[b]	0	<0.7	0	<1.1	0	0	11.8	8.9
	22[b]	0	<0.7	0	<1.1	0	0	10.2	10.6
		mean 0	mean <0.7	mean 0	mean <1.1	mean 0	mean 0	mean 12.0	mean 10.6
ts-1	23[b]	0	<0.7	0	<1.1	0	0	10.9	9.8
	29[b]	0	<0.7	0	<1.1	0	0	12.2	15.8
	25[b]	5	0.7	0	<1.1	0	0	7.7	9.9
	26[b]	5	0.7	0	<1.1	0	0	17.7	14.5
		mean 2.5	mean 0.7	mean 0	mean <1.1	mean 0	mean 0	mean 12.1	mean 12.5
none	9[c]	9	5.1	13	5.4	1.0	1	<3.3	12.4
	10[c]	9	6.0	8	6.0	1.7	4	<3.3	13.2
	11[b]	13	5.3	8	5.9	2.1	3	<3.3	11.6
	12[b]	9	5.4	8	5.6	1.0	3	<3.3	11.2
		mean 10	mean 5.5	mean 9.3	mean 5.7	mean 1.5	mean 2.8	mean <3.3	mean 12.1

[a]Mean rhinorrhea score represents the sum of scores during the eight days of peak virus shedding divided by eight. Four is the highest score; zero is the lowest score.

[b]Animals from Crowe, et al. Vaccine 1993, in press.

[c]Animals from Collins, et al., Vaccine 8: 164-168, 1990.

Table 17 Efficacy of plaque formation of *ts*-4 derivatives

Strain	Virus Titer (log_{10}pfu/ml)				
	32°	35°	36°	37°	38°
ts-4/F-15-8	6.0	5.9*	6.0*	<2.0[a]	<2.0
ts-4/F-19-1	5.5	5.3*	5.4*	<2.0	<2.0
ts-4/F-20-7	6.1	6.1*	6.1*	<2.0[a]	<2.0
ts-4/F-29-7	5.8	<2.0	<2.0	<2.0	<2.0
ts-4/F-31-2	5.6	<2.0[a]	<2.0[a]	<2.0	<2.0
ts-4 (Parent)	6.1	6.3	6.3	6.1	<2.0

a. Hazy stain.
*Small-plaque phenotype (<50% wild-type plaque size)

Human Studies

The attenuated virus of the invention is administered to human subjects according to well established human RS vaccine protocols, as described in, e.g., Wright et al., Infect. Immun. 37:397-400 (1982), Kim et al., Pediatrics 52:56-63 (1973), and Wright et al., J. Pediatr. 88:931-936 (1976), which are incorporated herein by reference. Briefly, adults or children are inoculated intranasally via droplet with 10^3 to 10^5 PFU of attenuated virus per ml in a volume of 0.5 ml. Antibody response is evaluated by complement fixation, plaque neutralization, and/or enzyme-linked immunosorbent assay. Individuals are monitored for signs and symptoms of upper respiratory illness. As with administration to chimpanzees, the attenuated virus of the vaccine grows in the nasopharynx of vaccinees at levels approximately greater than 10-fold or more lower than wild-type virus, and approximately 10-fold or lower when compared to levels of cpRSV or other incompletely attenuated parental strain. Subsequent immunizations are administered periodically to the individuals as necessary to maintain sufficient levels of protective immunity.

From the foregoing it will be appreciated that, although specific embodiments of the invention have been described herein for purposes of illustration and understanding, various modifications may be made without deviating from the spirit and scope of the invention. Accordingly, the invention is not limited except as by the appended claims.

WHAT IS CLAIMED IS:

1. A vaccine composition comprising, in a physiologically acceptable carrier, at least one attenuated respiratory syncytial virus having at least two attenuating mutations, that is selected from temperature-sensitive mutants of host range-restricted cold-passaged respiratory syncytial virus, cold adapted mutants of host range-restricted cold-passaged respiratory syncytial virus or temperature sensitive respiratory syncytial virus mutants having further temperature sensitive mutations.

2. The vaccine compositon according to Claim 1, which further comprises an adjuvant to enhance the immune response.

3. The vaccine composition according to Claim 1, wherein the virus is a host range-restricted cold-passaged respiratory syncytial virus which has been further attenuated by introducing one or more additional mutations which render the virus temperature-sensitive or unable to produce normal size plaques on tissue culture.

4. The vaccine of Claim 1, wherein the attenuated virus is a host range-restricted, cold-passaged respiratory syncytial virus which has been cold-passaged at temperatures of about 20°C to about 25°C to further attenuate the virus and render it cold adapted.

5. The vaccine of Claim 1, wherein the attenuated virus is ts-1 NG-1 which has been further attenuated by introducing one or more mutations by cold-passage or chemical mutagenesis which render the virus unable to produce normal size plaques on tissue culture or further temperature-sensitive.

6. The vaccine of Claim 5, wherein the ts-1 NG-1 virus is further attenuated by introducing at least one said mutation.

7. The vaccine of Claim 1, wherein the attenuated virus is ts-4 which has been further attenuated by introducing one or more mutations by cold-passage or chemical mutagenesis, which render the virus unable to produce normal size plaques on tissue culture or further temperature-sensitive.

8. The vaccine of Claim 7, wherein the ts-4 virus is further attenuated by introducing at least one said mutation.

9. The vaccine of Claim 1, wherein the attenuated virus is subgroup A or B.

10. The vaccine of Claim 1, wherein the attenuated virus is of an A2 strain of subgroup A.

11. The vaccine composition of Claim 1, wherein the attenuated respiratory syncytial virus is selected from the group:

(i) cpts RSV 248, 248/404, 248/804, 248/955, cpts spRSV 248/1228;
(ii) cpts RSV 475;
(iii) cpts RSV 530, 530/9, 530/346, 530/653, 530/667, 530/403, 530/188, 530/464, 530/1009, 530/1178, 530/1074, 530/963, 530/977, 530/1030, 530/1003;
(iv) cpRSV 3131 D1;
(v) RSV ts-1 NG1/A-20-4, A-37-8, A-15-7, A-25-8, A-21; and
(vi) RSV ts-4/F-15-8, F-19-1, F-20-7, F-29-7, F-31-2.

12. The vaccine composition of Claim 1, wherein the attenuated respiratory syncytial virus is selected from the group:

(i) cpts RSV 248, 248/404, 248/804, 248/955, cpts spRSV 248/1228;
(ii) cpts RSV 530;
(iii) cpts RSV 3131 D1;,
(iv) RSV ts-1 NG1/A-20-4, A-37-8; and
(v) RSV ts-4/F-19-1, F-29-7.

13. The vaccine composition of Claim 1, wherein the attenuated respiratory syncytial virus is selected from the group:

(i) cpts RSV 248, 248/404;
(ii) cpts RSV 530
(iii) RSV ts-1 NG1/A-20-4, A-37-8; and
(iv) RSV ts-4/F-19-1, F-29-7.

14. The vaccine composition of Claim 1, wherein the attenuated respiratory syncytial virus is selected from:

(i) cpts RSV 248/404;

(ii) cpts RSV 530; and

(iii) RSV ts-1 NG1/A-20-4.

15. The vaccine of Claim 1, formulated in a dose of 10^3 to 10^6 PFU of attenuated virus.

16. A method for stimulating the immune system of an individual to induce protection against respiratory syncytial virus, which comprises administering to the individual an immunologically sufficient amount of at least one attenuated virus selected from temperature-sensitive mutants of host range-restricted cold-passaged respiratory syncytial virus having at least two attenuating mutations, cold adapted mutants of host range-restricted cold-passaged respiratory syncytial virus, and temperature sensitive respiratory syncytial virus having further temperature sensitive mutations in a physiologically acceptable carrier.

17. The method of Claim 16, wherein the virus is a host range-restricted cold-passaged respiratory syncytial virus which has been further attenuated by introducing two or more mutations which render the virus temperature-sensitive or unable to produce normal size plaques on tissue culture.

18. The method of Claim 16, wherein the attenuated virus is a host range-restricted cold-passaged respiratory syncytial virus which has been cold-passaged at temperatures of about 20°C to about 25°C to further attenuate the virus and render it cold adapted.

19. The method of Claim 16, wherein the attenuated virus is ts-1 NG-1 or ts-4 which has been further attenuated by introducing one or more mutations by cold-passage or chemical mutagenesis which render the virus unable to produce normal size plaques on tissue culture or further temperature-sensitive.

20. The method of Claim 16, wherein the attenuated respiratory syncytial virus is selected from the group:

(i) cpts RSV 248, 248/404, 248/804, 248/955, cpts spRSV 248/1228;

(ii) cpts RSV 475;

(iii) cpts RSV 530, 530/9, 530/346, 530/653, 530/667, 530/403, 530/188, 530/464, 530/1009, 530/1178, 530/1074, 530/963, 530/977, 530/1030, 530/1003;

(iv) cpRSV 3131 D1;

(v) RSV ts-1 NG1/A-20-4, A-37-8, A-15-7, A-25-8, A-21; and

(vi) RSV ts-4/F-15-8, F-19-1, F-20-7, F-29-7, F-31-2.

21. The method of Claim 16, wherein the attenuated respiratory syncytial virus is selected from the group:

(i) cpts RSV 248, 248/404, 248/804, 248/955, cpts spRSV 248/1228;

(ii) cpts RSV 530;

(iii) cpts RSV 3131 D1;,

(iv) RSV ts-1 NG1/A-20-4, A-37-8; and

(v) RSV ts-4/F-19-1, F-29-7.

22. The method of Claim 16, wherein the attenuated respiratory syncytial virus is selected from the group:

(i) cpts RSV 248, 248/404;

(ii) cpts RSV 530

(iii) RSV ts-1 NG1/A-20-4, A-37-8; and

(iv) RSV ts-4/F-19-1, F-29-7.

23. The method of Claim 16, wherein the attenuated respiratory syncytial virus is selected from:

(i) cpts RSV 248/404;

(ii) cpts RSV 530; and

(iii) RSV ts-1 NG1/A-20-4.

24. The method of Claim 16, wherein the attenuated virus is administered to the individual in a amount of 10^3 to 10^6 PFU.

25. The method of Claim 16, wherein the attenuated virus is administered to the upper respiratory tract of said individual.

26. The method of Claim 25, wherein the attenuated virus is administered to the nasopharynx.

27. The method of Claim 25, wherein the attenuated virus is administered by spray, droplet, or aerosol.

28. The method of Claim 16, wherein the attenuated virus is administered to an individual seronegative for antibodies to said virus.

29. The method of Claim 16, wherein the attenuated virus is administered to an individual seropositive to said virus.

INTERNATIONAL SEARCH REPORT

International Application No PCT/US 93/03670

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all)[6]

According to International Patent Classification (IPC) or to both National Classification and IPC

Int.Cl. 5 C12N15/01; A61K39/155; C12N7/04

II. FIELDS SEARCHED

Minimum Documentation Searched[7]

Classification System	Classification Symbols
Int.Cl. 5	C12N ; A61K ; C07K

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched[8]

III. DOCUMENTS CONSIDERED TO BE RELEVANT[9]

Category *	Citation of Document, [11] with indication, where appropriate, of the relevant passages [12]	Relevant to Claim No.[13]
X	ARCHIVES OF VIROLOGY vol. 54, no. 1-2, 1977, pages 53 - 60 RICHARDSON, L. S. ET AL. 'Isolation and characterization of further defective clones of a temperature sensitive mutant (ts-1) of respiratory syncytial virus' see the whole document	1

* Special categories of cited documents : [10]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
03 AUGUST 1993	24 -08- 1993
International Searching Authority	**Signature of Authorized Officer**
EUROPEAN PATENT OFFICE	CHAMBONNET F.J.

INTERNATIONAL SEARCH REPORT

Inte onal application No.
PCT/US93/03670

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos..
because they relate to subject matter not required to be searched by this Authority, namely:

Remark: Although claims 16 to 29 are directed to a method of treatment of the human/animal body the search has been carried out and based on the alleged effects of the compound/composition.

2. [ ] Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. [ ] As all searchable claims could be searches without effort jusufying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.